REVIEW ARTICLE

Sorting and processing of secretory proteins

Philippe A. HALBAN and Jean-Claude IRMINGER

Laboratoires de Recherche Louis Jeantet, Centre Médical Universitaire, 1 rue Michel Servet, 1211 Geneva 4, Switzerland

INTRODUCTION

The vast majority of secretory proteins in eukaryotic cells share a common biosynthetic origin in the rough endoplasmic reticulum (RER), from where they are transported to the Golgi complex. It is in the *trans*-Golgi network (TGN) that proteins destined for the regulated secretory pathway will be sorted from those to be secreted via the constitutive pathway. Both of these pathways involve vesicular transfer to the plasma membrane followed by the secretory event itself, exocytotic discharge of vesicle contents. In many instances, secretory proteins, even after cleavage of the signal peptide in the lumen of the RER, are in the form of a higher-molecular-mass precursor or proprotein. Conversion of proprotein to protein occurs in both the regulated and the constitutive pathways, but in different compartments and as a result of the action of different endoproteases. Sorting in the TGN and conversion of proproteins will be the major focus of this review.

PROTEIN SECRETORY PATHWAYS: A GENERAL OUTLINE

Leader and leaderless secretory proteins

Most secretory proteins are synthesized as precursors carrying a leader, or signal, peptide. It is the interaction of the signal peptide with its cognate protein complex (SRP or signal recognition particle) on the ribosome which assures that secretory proteins are synthesized on the RER and translocated into the lumen of this organelle, most typically as translation proceeds [1–5]. The cleavage of the signal peptide by signal peptidase occurs rapidly thereafter. It is in the RER that secretory proteins will become folded into their tertiary structure, assisted by chaperone proteins [6–11]. Oligomerization also occurs in this compartment [8]. For many secretory proteins, glycosylation is initiated within the RER and proceeds up to the *trans*-Golgi. Although glycosylation is not mandatory for secretory proteins (a well studied example of a non-glycosylated secretory protein being proinsulin), the study of mammalian glycosylation mutants has greatly facilitated the elucidation of the early steps in the secretory pathway [12].

There is a quality control procedure that ensures that proteins which are in some way damaged or incompletely folded, or have failed to oligomerize correctly, are not transported out of the RER [6,8]. Such altered proteins are typically degraded in the pre-Golgi degradation compartment [13,14].

Over the past few years, it has become apparent that an alternative to this classical pathway for secretory proteins exists in several, and perhaps all, cell types [15–17]. This novel pathway is employed by secretory proteins which lack a conventional hydrophobic signal sequence, including basic fibroblast growth factor and interleukin-1. Although the precise mechanism of secretion remains to be characterized, it appears that such proteins may be secreted directly from the cytosol via either ATP-dependent plasma membrane transporters or translocators [17], or as a result of localized evagination of the plasma membrane [18]. As for the classical secretory pathways (see below), secretion of proteins by these non-classic pathways can also be polarized [19].

Regulated and constitutive secretory pathways

Secretory proteins are transferred from the RER to the *cis*-Golgi, and from one Golgi stack to the next, in shuttle vesicles, finally reaching the TGN [20–22]. The elaboration and imaginative use of cell-free systems for the study of intracellular trafficking events has galvanized this field of cell biology, leading to rapid advances in our understanding of the molecular basis of these events [22–28]. The genetic approach to the study of trafficking and sorting has allowed for the identification of a myriad of proteins implicated in these events in yeast [29], and it has already proved possible in a few instances to identify the mammalian counterpart protein.

It is in the TGN [30] that proteins destined for the regulated pathway will be actively sorted from those to be released by the constitutive pathway (as discussed below in greater detail). The general features of the regulated pathway were described in a series of classic papers by Palade and co-workers in the late 1960s and early 1970s (reviewed by Palade himself in [31]). Although the distinction between the regulated and the constitutive pathways had already been made apparent by others [32,33], it was Kelly and co-workers who clearly demonstrated the co-existence of the two secretory routes for discrete subclasses of proteins within the same cell [34,35]. These two secretory pathways have been the focus of numerous review articles, including [20,35–40], and only their salient features will be summarized here.

As implied by the nomenclature, secretion via the regulated pathway can be modulated by secretagogues. This occurs at the level of exocytosis itself, the most distal step in the pathway, and allows for the rapid and massive discharge of proteins stored in granules at the physiologically appropriate moment. Exocytosis from the constitutive pathway is a continuous process limited only by the availability of product. Release from this pathway is thus only regulated at the most proximal level, i.e. biosynthesis. The other fundamental difference between these two secretory pathways lies in the kinetics of secretion. Whereas constitutive secretion occurs at an essentially constant rate, with transit from the TGN to the plasma membrane taking some 10 min [41,42], regulated secretory proteins can be stored for considerable periods of time in granules before their release is stimulated by a secretagogue. Release of preformed products from the regulated pathway is further distinguished by being dependent upon protein synthesis, whereas the constitutive pathway is not [43]. The generation of both constitutive vesicles and secretory granules of the regulated pathway is, however, GTP-dependent [44–46].

Aside from ensuring the secretion of all proteins not targeted

Abbreviations used: ACTH, adrenocorticotropic hormone (corticotropin); CLIP, corticotropin-like intermediate lobe peptide; C-peptide, connecting peptide; JP, joining peptide; LPH, lipotropic hormone (lipotropin); MSH, melanocyte-stimulating hormone (melanotropin); NGF, nerve growth factor; POMC, pro-opiomelanocortin; RER, rough endoplasmic reticulum; TGN, *trans*-Golgi network; vWF, von Willebrand factor.

to the regulated pathway, the constitutive pathway is responsible for delivery of integral membrane proteins to the cell surface. Both secretion and delivery of proteins to the plasma membrane are very much more complex in polarized cells [47–49]. Polarization is an issue not only for the constitutive pathway, but also for regulated secretion from both epithelial cells and, most notably, exocrine cells [20,40], as well as from some endocrine cells, including the pancreatic B cell [50].

In neuronal and some endocrine cells, chemical neurotransmitters are sequestered into neurosecretory vesicles and discharged in response to a stimulus. There are thus two unrelated and functionally distinct regulated secretory pathways [51–54]. The secretion of neurotransmitters will not be discussed further here.

Immature and mature secretory granules

Those regions of the TGN destined to form granules, as well as the earliest, immature form of the secretory granule of the regulated pathway, are coated with clathrin [55–61]. Clathrin [62] has been implicated in secretory, endocytotic and lysosomal trafficking pathways, although its precise role remains obscure [63–66]. It forms a basket-like web of triskelions [67], composed of the clathrin heavy and light chains, on the cytosolic face of membranes [66]. There are two light chains, LCa and LCb, both of which exist in two forms of different molecular mass due to alternative splicing of their mRNA, the higher-molecular-mass forms being restricted to neurons [68]. It has been found that, in cells which have the regulated secretory pathway, the LCb chain predominates [69], although it is not yet known whether this is related to clathrin coating of the immature granule. In yeast, clathrin is involved in the retention of the Kex2 conversion endoprotease in the Golgi complex [70].

The immature, clathrin-coated granule is the compartment in which the bulk of prohormone conversion occurs (see below). The maturation of secretory granules involves not only progressive acidification and prohormone conversion but also the loss of the clathrin coat [71,72]. Clathrin is removed from coated vesicles by disassembly mediated by an ATP-dependent uncoating enzyme [73] shown to be a member of the 70 kDa family of stress proteins [74]. It is not yet known whether the same mechanism is responsible for the uncoating of secretory granules.

The imaginative use of a cell-free system and subcellular fractionation techniques has allowed the generation and physical separation of immature secretory granules from both mature granules and the TGN [75,76]. In the PC12 cells used in these particular studies, maturation of the secretory granule involved an increase in size which is presumed to be due to the fusion of immature granules. Although possibly typical of neuronal cells, this may not be the case in other regulated cell types. Indeed, if the refinement of granule contents implicit in the post-granular sorting pathways discussed in the following section occurs, one should expect a decrease in size upon granule maturation, and this is indeed what is found in the granules of pancreatic endocrine cells (M. Neerman-Arbez and P. A. Halban, unpublished work).

Alternative secretory pathways: sorting from the regulated pathway to the post-granular 'constitutive-like' pathway

Although the targeting of proteins to the regulated pathway was initially believed to be the last sorting event prior to secretion, Castle, Arvan and co-workers [77–79] have proposed that a refinement of secretory granule contents can occur during granule maturation, as reviewed in [80–82]. They suggest that small regions of maturing granules can pinch off to form secretory vesicles, carrying within them a random sampling of any constituent that was soluble in the granule interior. These vesicles will rapidly release their contents by exocytosis in a constitutive fashion. This pathway has therefore been referred to as a post-granular 'constitutive-like' secretory route. Although first described in exocrine cells [77,78] it has since been observed in endocrine cells as well [83–86] and must therefore be regarded as an integral and perhaps obligatory feature of this pathway. Secretion is just one possible fate for the contents of the vesicles in question. Indeed, in quantitative terms, secretion via this pathway is only of modest importance, accounting for the release of no more than some 10% of newly synthesized soluble proteins originally located in secretory granules [84,86]. It is attractive to speculate that the post-granular vesicles could shuttle soluble granule products back to the TGN or to lysosomes [82,85,86].

SORTING OF PROTEINS DESTINED FOR THE REGULATED SECRETORY PATHWAY

The TGN, as the most distal compartment common to both the regulated and the constitutive pathways, is the sorting compartment

As described above, all secretory proteins which initially present a signal sequence and are sequestered into the lumen of the RER are routed to the Golgi complex and, after intercisternal transport, arrive in the TGN [22,30] (Figure 1). This is one of the major protein sorting compartments of the cell, ensuring not only segregation of proteins destined for regulated or constitutive secretion, but also targeting of proteins to lysosomes and retention of Golgi proteins. Ironically, for the purposes of this review, more is known about the molecular mechanism of these other sorting events than about targeting to the regulated pathway. Thus proteins are recognized and segregated in the TGN for dispatch to lysosomes by virtue of their binding to mannose 6-phosphate receptors [87–92]. The mechanism for local retention of integral *trans*-Golgi membrane proteins is less well defined, but does appear to depend upon unique domains on the cytosolic face of such proteins which interact with select cytosolic proteins presumably serving as retention signals [93].

Evidence for the central role of the TGN in the segregation of regulated and constitutive secretory proteins stems from both morphological and biochemical studies. It was thus apparent, even from early studies on exocrine cells, that the Golgi complex was the most distal compartment in which proteins destined for constitutive release or for packaging in zymogen granules were co-localized [31]. Subsequent studies showed that passage through the *trans*-Golgi was an obligatory step in secretion [32,94]. More recent morphological studies, in which the intracellular trafficking of regulated and constitutive secretory proteins was followed in parallel, have confirmed and extended these early observations [95,96]. The identification of the TGN as the last compartment common to both secretory pathways is supported by the pattern of glycosylation [97,98].

Sorting to the regulated pathway is the active segregation event; the constitutive pathway is a default, bulk flow, route

Even though it was clear from the studies just described that the two classes of secretory proteins parted ways, and were thus sorted one from the other, in the TGN, it was far from clear how the sorting occurred, or indeed whether an active sorting process was implicated in targeting to the regulated or to the constitutive pathway, or perhaps to both. To answer this central question, Kelly and colleagues expressed a regulated protein (growth hormone), a constitutive protein (the ectodomain of a viral

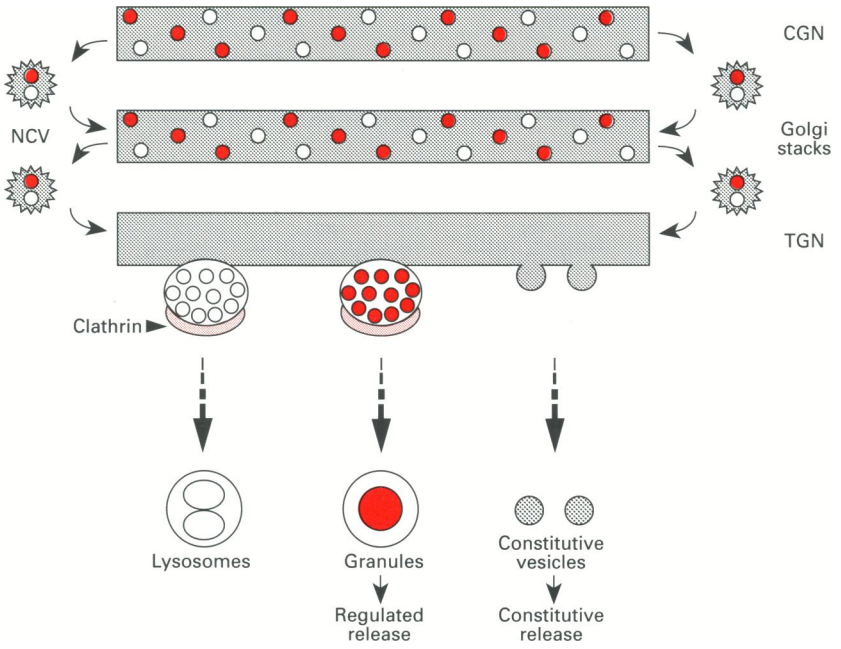

Figure 1 The TGN: the cellular sorting house

For simplicity, only secretory and lysosomal proteins are portrayed (to the exclusion of proteins destined for retention in Golgi compartments). Integral plasma membrane proteins are handled like soluble proteins released by the constitutive pathway. Proteins received from the RER are transferred from the *cis*-Golgi network (CGN) to the TGN via the Golgi stacks in non-clathrin-coated vesicles (NCV). In the TGN, lysosomal proteins and regulated secretory proteins are actively sorted to corresponding clathrin-coated regions. Delivery to the constitutive vesicles (which may be non-clathrin-coated) is presumed to be by default. Lysosomal proteins are thought to be delivered first to a late endosomal compartment before reaching their ultimate destination (not shown). Immature granules still carry a partial coating of clathrin; only mature granules are shown here.

glycoprotein) or a hybrid protein (the viral protein fused to the C-terminal domain of growth hormone) in AtT20 cells (transformed pituitary corticotrophs) [99]. The viral protein was, as expected, released via the constitutive pathway, but when fused to part of growth hormone it was diverted to the regulated pathway. These data suggested for the first time that regulated proteins carry within their structure domains which serve as recognition signals for the sorting process, and that the sorting mechanism can be dominant [20,99]. The principle of the dominance of regulated over constitutive proteins was confirmed by Huttner and co-workers in a study in which antibodies to either a regulated (secretogranin I) or a constitutive (G protein of vesicular stomatitis virus) protein were expressed in PC12 cells [100]. The anti-secretogranin antibody was diverted to the regulated pathway along with its antigen. Interestingly, in this experimental setting, the 'hijacked' constitutive protein was associated only by non-covalent forces to the 'hijacker' (the regulated protein). Support for the constitutive pathway being a default route stems from studies in which it was shown that molecules presumed to be too small or primitive to carry structural domains that would be recognized by any sorting machinery (a glycosaminoglycan [101] and a tripeptide [41] respectively) were secreted via this pathway.

Sorting is pH-dependent

In 1983 [102] it was shown that, when AtT20 cells were incubated in the presence of chloroquine, a drug believed to neutralize acidifying compartments, POMC (pro-opiomelanocortin), the precursor to adrenocorticotropic hormone (ACTH), which is the major peptide released through the regulated pathway of these cells, was diverted to the constitutive pathway. This led to the hypothesis that the sorting process is favoured by an acidic environment. These results and the hypothesis itself have, however, been contested on theoretical grounds [103]. Furthermore, although similar results (i.e. diversion of regulated proteins to the constitutive pathway) were found when PC12 (pheochromocytoma) cells, for example, were incubated in the presence of ammonium chloride [104], others have obtained divergent results in exocrine cells [105] and even in AtT20 cells [106].

Despite these conflicting views, the present consensus does seem to lean towards pH-dependency of the TGN sorting event. This is indeed supported by estimates of the pH of the TGN. Using an immunocytochemical approach, Anderson and co-workers have estimated a pH of approx. 6.5 for cisternae of the *trans*-Golgi [107]. Such acidification is due to the activity of an ATP-dependent proton pump [108–110], and is most certainly important for preserving TGN function as a whole [111,112].

Sorting is an active process leading to refinement and concentration of granule constituents relative to the TGN

As discussed in detail below, secretory proteins appear to condense in the TGN. Condensation is presumed to be important for sorting to the regulated pathway and is the key event responsible for the remarkably high local concentration of regulated secretory proteins in granules, estimated as 10 [113] or even 60 mM [114] for some hormones, corresponding to thousands or tens of thousands of hormone molecules per granule. Depending upon the protein in question, the increase in secretory protein concentration in the granule compared with more proximal compartments in the secretory pathway can vary from ten to several hundred fold [35]. Such concentration in granules allows in turn for the quantal release of large amounts

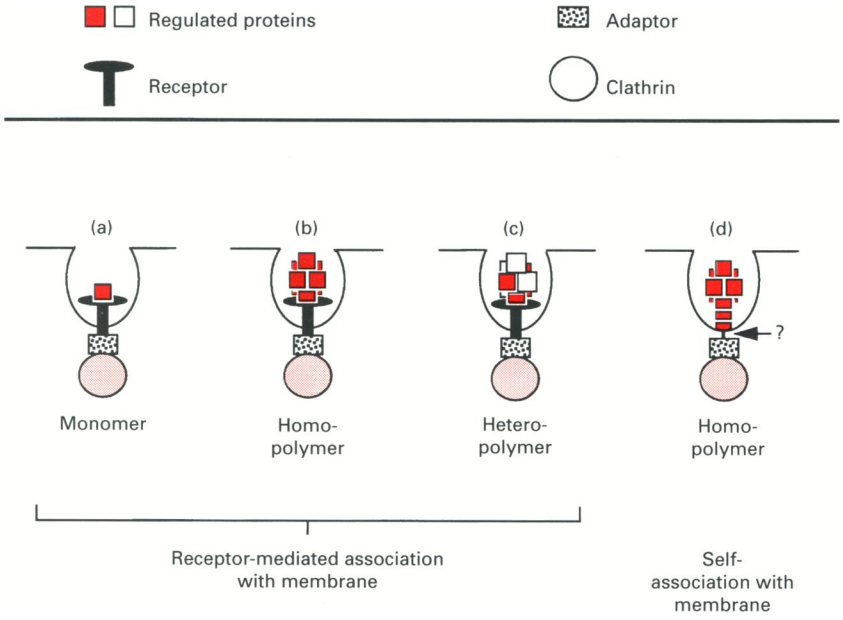

Figure 2 Possible mechanisms for the sorting of proteins to the regulated pathway

Each model is discussed in detail in the text. In models (**b**) and (**c**), the receptor could recognize a monomer initially, which could then serve as the nucleus for the aggregation event. Alternatively, the receptor may only recognize aggregates. In model (**c**), the white protein alone (whether monomeric or aggregated) may not be recognized by the receptor; it becomes bound by virtue of its association with the red protein, which thus serves as a chaperone in this context. In model (**d**), the intermediary assuring binding of clathrin adaptors has yet to be proposed (as indicated by the question mark); in the other models, the adaptors can bind to the cytosolic tail of the putative receptor.

of protein for each single exocytotic event, a hallmark of the regulated pathway.

POSSIBLE MECHANISMS FOR SORTING OF REGULATED PROTEINS IN THE TGN AND THEIR TARGETING TO GRANULES

As outlined in Figure 2, even though the sorting mechanism responsible for directing proteins to the regulated pathway remains to be identified, a number of interesting models have been proposed. As will become apparent, there is considerable overlap between these models, and in reality the sorting process probably involves aspects of each. Secretory granules of the regulated pathway carry many proteins aside from regulatory peptides or zymogens [113,115–119]. These other proteins (both soluble and transmembrane) are of central importance not only to granule integrity but also to granule function and to the secretory process itself. All granule proteins are presumably subject to a sorting and targeting process in the TGN.

Receptor-mediated sorting and targeting of proteins to granules

The formation of secretory granules and receptor-mediated endocytosis of a surface-bound hormone share many features in common [55], including the budding and formation of clathrin-coated vesicles carrying only the appropriate proteins that have been selected from the many others available in the donor compartment (TGN or plasma membrane respectively). Based upon this observation, it was postulated [55] that the active sorting process required for targeting to the regulated pathway is receptor-mediated. The intimate association of proinsulin with the inner face of Golgi membranes in pancreatic B-cells [120] can be considered as further experimental evidence for a receptor-mediated sorting event in the TGN (although in this study it was not possible to determine in which Golgi compartment the proinsulin was bound). As noted by Orci et al. [120], however, proteins secreted via the constitutive pathway have also been shown to bind to Golgi membranes [121], yet they are not believed to be subject to an active sorting process.

The functional characteristics demanded of the putative sorting receptors became much clearer once it was possible to express foreign secretory proteins in regulated cells by transfection [122]. Using such an approach, it was shown that a foreign protein such as proinsulin can be correctly sorted to secretory granules and processed to insulin in pituitary corticotroph AtT20 cells which normally synthesize and sort an unrelated prohormone, POMC [123]. The many other examples in the literature of the targeting and processing (when pertinent) of foreign peptide hormones are too numerous to cite, but include the following early, ground-laying, studies: growth hormone in AtT20 cells [97]; pro-somatostatin in GH3 (pituitary) cells [124]; proneuropeptide Y in AtT20 cells [125]; proparathyroid hormone in AtT20 cells [126]; proenkephalin in AtT20 cells [127]. Indeed, even exocrine enzymes such as trypsinogen can be recognized by the sorting machinery of endocrine cells [128]. Given the extraordinary number of molecules delivered to the granules of the regulated pathway at any given time (when synthesis is stimulated) [115,118], and given this apparent diversity in terms of substrate specificity, it became increasingly unlikely that discrete receptors existed for each and every regulated protein, and that all types of regulated cells expressed all such receptors spontaneously. Rather, it is now believed that if sorting receptors do indeed exist, they must have extremely broad specificity, with, possibly, just one class of receptor recognizing all proteins destined for the regulated pathway. Despite efforts to identify such receptors [129], if indeed they exist, they remain elusive.

If sorting is dependent upon binding to receptors then, it has been reasoned, there must be structural domains on regulated proteins, such as proinsulin, which are recognized and bound by these receptors [72,130,131]. Several groups have therefore em-

barked on the structural analysis of regulated proteins in order to identify such domains. Two approaches have been used. In the first, structural alterations are introduced into the protein by site-directed mutagenesis and the modified protein is expressed in a regulated cell. The impact of the modification on sorting can then be examined. This approach is labour intensive and time-consuming. Another major drawback is that the three-dimensional structures of most proteins targeted to the regulated pathway remain to be elucidated. It is therefore more often than not impossible to predict the impact of the modification or deletion of a linear array of amino acids on three-dimensional structural domains, and it is thus difficult to interpret the data obtained from such experiments. Despite these obstacles, the approach has been applied with some success to somatostatin in particular. It has been shown that the pro-region of this prohormone carries the domain for sorting to the regulated pathway [132,133]. If this region is deleted, the truncated molecule is released via the constitutive pathway. If this same region is linked to α-globin, a cytosolic protein, it serves as a dominant sorting signal and the hybrid molecule is delivered to the regulated pathway [134]. This particular pro-region consists of 78 residues and these studies shed no further light on the precise structural domain or feature recognized by the sorting machinery. Indeed, studies on the targeting of prosomatostatin molecules in transfected RIN (insulinoma) cells have revealed an additional level of complexity, with multiple sorting signals leading to targeting to two alternative regulated pathways [135]. The sorting mechanism for integral membrane proteins of the granule has also been studied in this way, and appears to be quite different from that for soluble proteins. Thus P-selectin, a transmembrane protein localized to granules in endothelial cells, is directed to granules in AtT20 cells by virtue of a cytoplasmic domain [136].

The second approach for identifying domains implicated in protein sorting to the regulated pathway depends upon the comparison of sequences and postulated ordered three-dimensional structures of all proteins destined for this pathway. Kizer and Tropsha [137] used this approach, combined with some additional theoretical refinements, to generate a sorting domain common to all proproteins known to be targeted to the regulated pathway of AtT20 cells, but absent from those mutant proproteins which fail to be correctly targeted. They propose a somewhat degenerate amphipathic helical sorting sequence reminiscent in some respects of those suggested to be involved in targeting to the RER or to mitochondria. This putative sorting domain has yet to be tested experimentally. It must be noted that even if a sorting domain can be identified in a regulated secretory protein, it could either be recognized by the putative sorting receptors or be required for some condensation or aggregation event implicated in sorting. The mere identification of the existence of sorting domains may not therefore shed much light on the sorting mechanism itself.

Aggregation as the initial sorting event

Condensation of secretory proteins in a late Golgi compartment was noted in the early studies of Palade on exocrine cells [31]. This seems to be one hallmark of cells expressing the regulated pathway, and has led to the concept of condensation as the primary sorting event [35,40]. The concept is at first sight elegant in its simplicity. Secretory proteins destined for the regulated pathway are proposed to be endowed with the ability to aggregate as soon as a critical local concentration is reached. The concentration needed for aggregation may change depending upon the local environment, and in particular on the concentration of Ca^{2+} and the pH, as discussed below. Thus, whereas the TGN is the usual site of aggregation, it can also occur in proximal compartments, including the RER [138,139] if the rate of synthesis of the secretory protein greatly outpaces the rate of exit from the given compartment. Even under such unique circumstances, the condensation event leads to sorting of secretory products from other (in this case resident ER) proteins [139].

The example of the secretogranins

The condensation-sorting model is supported not only by morphological evidence but also by more direct studies on the physico-chemical characteristics of some proteins found in the regulated pathway and, in particular, their ability to aggregate under conditions thought to mimic those encountered in the TGN. The best studied example is the family of secretogranin proteins [140–142]. These acidic secretory proteins are found within secretory granules of the regulated pathway of many cell types, and present several attractive features for studying the sorting mechanism of the regulated pathway [141,142]. Although their biological function is not yet well established, it has been suggested that they may play a central role in facilitating protein sorting to the regulated pathway as well as serving as precursors for biologically active peptides (i.e. the generation of pancreastatin by proteolytic cleavage of chromogranin A) [143,144]. *In vitro*, secretogranin II has been shown to aggregate and precipitate when the pH was lowered to 5.2 and the Ca^{2+} concentration raised to 10 mM [104]. When the granin was mixed with a constitutively secreted protein (IgG) there was sorting of the two, since the IgG remained in solution in the face of the precipitable granin. The other granins also display Ca^{2+}-dependent aggregation [145,146], and have been shown to form co-aggregates with another regulated protein, parathormone [146]. Chanat and Huttner [147] extended their study of granins to a cell-free system in which these proteins were shown to remain in an insoluble state within the lumen of semi-permeabilized TGN vesicles, providing that the Ca^{2+} concentration exceeded 1 mM, and at a pH of 6.4.

These studies on the behaviour of granins *in vitro* are, of course, only of relevance if the conditions used for aggregation faithfully reproduce those encountered in the sorting compartment. The best estimates of both the luminal pH and the $[Ca^{2+}]$ in TGN would appear to match the values used in the study of Chanat and Huttner [147]. The pH, shown to be slightly less than neutral, yet higher than that found in granules, can thus be presumed to be around 6.4 [30,107,111,112,148,149], with the $[Ca^{2+}]$ certainly in the region of 10 mM. There may well, however, be significant differences from one cell type to the next, and one can readily imagine fluctuations in these values within a given cell. Estimating the true local concentration of regulated proteins within the TGN also poses a serious problem. Even if the mean concentration within this compartment can be calculated, the local concentration within a specific region, or subcompartment, may be quite different.

Despite these potential complicating factors, the work on the granins does offer a credible working model for condensation-sorting of regulated proteins in the TGN.

Aggregation of other regulated secretory proteins: formation of homo- or hetero-aggregates

Although the secretogranins have the ability to aggregate in a low-pH and high-Ca^{2+} environment, this is in all probability a feature common to some, but not to all, proteins destined for the regulated pathway. Indeed, the unique acidic domains of the granins which are implicated in the precipitation event are not found in all regulated proteins. We are therefore left to speculate

that for some proteins, which cannot spontaneously form aggregates on their own, the condensation-sorting process, if it applies to such proteins, must depend upon their interaction and co-aggregation with proteins which do aggregate in the TGN. This latter class of proteins, serving as seeds for heteroaggregates, possibly includes secretogranins as well as two other proteins shown to promote pH- and Ca^{2+}-induced aggregation of regulated proteins *in vitro* (chymotrypsinogen [150] and GP-2 [151]).

It must thus be envisaged that both homo- and hetero-aggregates may be formed in the TGN during the condensation-sorting event, and thereafter in granules, and there is direct experimental evidence for this [152]. The affinity of regulated proteins for themselves and for others within the TGN would lead to intrinsic, albeit non-random, heterogeneity of aggregates formed in the TGN. This in turn could account for the diversity of secretory granule contents within the very same cell (see for example [153,154]), as well as the generation of morphologically distinct granule forms upon expression of a foreign protein such as von Willebrand factor (vWF) in AtT20 cells [155].

Targeting of aggregates to granules: self-driven or receptor-mediated

The formation of aggregates in the TGN provides an attractive model for the sorting of regulated from constitutive proteins, and for the local concentration of regulated proteins observed by electron microscopy. As yet unanswered is whether the formation of aggregates induces granule formation *per se*, or whether other membrane proteins are involved, serving the role of receptor and guide. Sorting receptors have already been discussed in some depth above. Whether the receptors recognize soluble monomers or precipitable aggregates within the TGN does not affect their intrinsic function, which is to recognize proteins destined for the regulated pathway and to deliver them to nascent granules.

There exists a very real possibility that regulated proteins may themselves serve as sorting receptors. Targeting would in this case be a 'self-drive' (homophilic) phenomenon, and as such integrated with condensation-sorting itself, rather than 'chauffeur-driven' (mediated by a receptor unrelated to the secretory proteins found in the aggregates). This hypothesis is based upon the intriguing observation by Huttner and colleagues that chromogranin B exists in a tight membrane- associated form in PC12 cells [156] and is even presented at the cell surface in this form in a transient fashion. They propose that some molecules of the chromogranin bind to the inner face of TGN membranes, serving as the nucleus for aggregation. Although the precise mechanism for the association of chromogranins to the membrane remains obscure, in the case of chromogranin B the reduction of an intramolecular disulphide loop leads to mis-sorting of the protein to the constitutive pathway in PC12 cells [157]. Since reduction did not affect aggregation, the authors suggest that it was association with the TGN membrane that was perturbed. This does not, however, appear to be a universal mechanism, and is not even common to all granins, since secretogranin II, which does not feature this disulphide loop, was normally targeted to granules even following reduction.

The phenomenon does not seem to be limited to homo-aggregates of chromogranins, since other granule constituents have been found in a membrane-associated form [152]. Most recently, Leblond et al. [151] have shown that GP-2, a protein found as a major constituent in possibly all granules, facilitates zymogen aggregation *in vitro*. GP-2 is found both in a soluble form as well as attached to the membrane by a glycosyl-phosphatidylinositol anchor. It could thus serve as the targeting domain of a heteroaggregate.

Clathrin coating of immature granules and the problem of adaptors (adaptins)

As mentioned above, the earliest, immature, forms of secretory granules carry a partial coating of clathrin on their cytosolic face [55–58,61,71]. Clathrin assembly depends upon the association of specialized adaptor proteins (adaptins, or assembly proteins) [158,159] with the cytosolic tail of select transmembrane proteins (typically receptors following binding of their ligand). The clathrin chains will in turn form triskelions by association with the adaptins [66,158,159].

Based upon this knowledge, it must be assumed that adaptins are attracted to those parts of the TGN involved in granule budding. If so, it must be further assumed that the adaptins bind to transmembrane proteins concentrated in these TGN membrane domains. An attractive candidate for such a transmembrane protein would be the putative sorting receptor. If the self-association of aggregates lies at the heart of the TGN targeting process, then one must imagine that this association in some way leads to an interaction and recruitment of integral TGN proteins, which will serve as the adaptin attractant.

INTRACELLULAR PROCESSING OF SECRETORY PROTEIN PRECURSORS BY LIMITED PROTEOLYSIS (CONVERSION OF PROPROTEINS)

Post-translational modification of one form or another is central to the synthesis of the fully active form of almost all proteins. Such modifications include phosphorylation, glycosylation and sulphation of amino acid side-chains, as well as α-amidation of C-terminal residues (a modification of particular importance for a variety of neuropeptides [160]). Another fundamental example is protein processing by limited proteolysis, which can be subdivided into three classes: (1) removal of signal peptide of preproteins by signal peptidase in the RER; (2) limited proteolysis of proteins after their release from the cell of origin, a classic and historic example being the activation of zymogens, comprehensively reviewed more than 35 years ago by Neurath [161]; and (3) intracellular processing of proproteins, which is the focus of this review.

Although alluded to earlier [162], it was in 1967 that Steiner provided the first direct evidence for the processing of a higher-molecular-mass precursor, proinsulin, into a smaller, biologically active peptide hormone, insulin [163,164]. Since that time it has become apparent that the vast majority of regulatory peptides are derived from a higher-molecular-mass precursor, or proprotein, by limited proteolysis, a process now known as proprotein conversion. Of the many excellent reviews on the subject, several are notable for their historical perspective and discussion of the biological importance of conversion [165–171]. More specialized reviews, dealing more specifically with the enzymology of conversion, will be cited in context below.

The biological relevance of proprotein conversion

Proprotein processing leads to the generation of at least two, and frequently many more, smaller peptides from limited proteolysis of a single precursor. In many instances, conversion of a multifunctional precursor gives rise to a host of biologically active peptides, and is as such an example of a biological cascade providing for functional diversity. The added sophistication of cell-specific expression of conversion endoproteases allows for alternative pathways of conversion (differential processing), an important component in the elaboration of cellular differentiated function. The exemplar molecule in this context is POMC, a proprotein which encompasses a number of small-molecular-

mass regulatory peptides, discrete subpopulations of which can be selectively generated in a tissue-specific fashion by differential processing [33,172] (see Figure 4). Although less complex, the differential processing of prosomatostatin has also provided an interesting model system for studying prohormone processing mechanisms [133,173,174].

In almost all instances, at least some of the peptides generated by conversion do not have any known biological function as secretory products. A better understanding of intracellular sorting and conversion events has, in some instances, attributed a role for these peptides while they are still retained within the precursor molecule, two examples being the postulated involvement of the pro-region of prosomatostatin in targeting to the regulated pathway ([134] and see above) and that of the connecting peptide (C-peptide) of proinsulin in proinsulin conversion [175]. Additional functions have been suggested for pro-regions that are as yet without any known function. These include helping the precursor to fold into its correct three-dimensional structure, or simply as a spacer peptide assuring the minimum length believed to be necessary for a preprotein to penetrate into the lumen of the RER [166].

Discussion of proprotein conversion will be divided into two sections, dealing with the conversion compartment and conversion enzymology respectively.

CELLULAR COMPARTMENTS FOR PROPROTEIN CONVERSION

The regulated pathway: the immature, clathrin-coated granule

The immunolocalization of proinsulin, proinsulin conversion intermediates or insulin in the pancreatic B-cell by Orci and colleagues has shown that the immature, clathrin-coated granule is proinsulin-rich whereas, conversely, the mature, uncoated granule is insulin-rich and proinsulin-poor [58,60,71,176,177]. This provides convincing evidence for the former as the conversion compartment in B-cells. It was further shown that, in these cells, even though the immature granule is mildly acidic, there is further acidification as the granule matures [71,176] (although intriguingly, such is not the case in exocrine cells, in which zymogen granules are less acidic than their precursor, the condensing vacuole [178]). Such acidification of the granule milieu (which is due to the granule ATP-dependent proton pump [179,180]) would certainly be expected to promote conversion, since the putative conversion endoproteases both display an acidic pH optimum [181]. Indeed, inhibition of the granule proton pump, which will result in increased intra-granular pH, inhibits proinsulin conversion in purified granules [180].

There has been speculation that some limited conversion of proinsulin could arise in the TGN [181]. If such a precocious event does occur, it is extremely limited in quantitative terms [177]. It will be seen below that the conversion of constitutive proteins may well occur in the TGN. If this is the case, then it is quite possible that any regulated peptide presenting a cleavage site susceptible to proteolytic attack by the convertases of the constitutive pathway may well experience some cleavage while in transit in the TGN, the kinetics of which should be quite distinct from those pertaining to conversion by PC1 or PC2 in secretory granules.

Granules have been shown to be the compartment for conversion of other prohormones, including POMC [182,183], although there are again those who would favour the TGN as the compartment for the earliest conversion events [184].

In keeping with a central role of granules in conversion, the enzymes responsible for prohormone conversion are found within secretory granules [185–187]. Other enzymes implicated in later, post-cleavage processing events, such as carboxypeptidase H/E (removal of C-terminal basic residues after endoprotease cleavage) and PAM (peptidyl α-amidating mono-oxygenase), have similarly been localized to granules [188].

In conclusion, the weight of evidence suggests that even if limited conversion of proproteins destined for the regulated pathway can arise in the TGN, the bulk occurs in granules. Any conversion, or partial conversion, which may occur in the TGN could be due to the action of the constitutive pathway conversion enzymes or to activity of the regulated pathway enzymes before their packaging into granules. Very little is known concerning the trafficking and compartmentalization of the conversion endoproteases, and this will be an interesting area to follow over the next few years.

The constitutive pathway: TGN or the constitutive secretory vesicle?

It is now known that proprotein conversion in the constitutive pathway is a relatively common event, at least for secretory proteins. For receptors, which must be delivered to the cell surface in constitutive secretory vesicles, conversion is the exception rather than the rule [189].

The very earliest studies on conversion in the constitutive pathway focused on proalbumin, at a time when the Kex2-like family of mammalian conversion endoproteases had yet to be identified. The existence of an albumin precursor became apparent in 1973 [190], quite early in the history of proprotein studies. It was immediately apparent that if liver cells secreted large amounts of albumin, relatively little of either the mature or precursor protein was to be found inside the cells (in keeping with a cell secreting via the constitutive pathway). The use of inhibitors which prevented the discharge of secretory vesicle contents, or which prevented their formation, combined with subcellular fractionation, indicated that conversion was a very late event, occurring just before release. The compartment was identified as the secretory vesicles by some [191–193], and as the Golgi complex by others [194]. In another study it was suggested that proalbumin and the conversion enzymes may be directed to separate vesicles which must fuse before conversion can proceed [195].

As discussed in greater detail below, it is now believed that the conversion endoprotease of the constitutive pathway is furin, PACE 4 or another closely related enzyme. The generation of antibodies directed against such proteins has allowed for their localization by immunocytochemistry. The consensus arising from such studies is that these enzymes are to be found in the Golgi complex (see, for example, [196,197]). Note, however, that constitutive vesicles are small, and by definition there are few to be found in a cell under physiological circumstances (the transit time from the TGN to the plasma membrane being extremely rapid). Neither conventional (light microscopy) nor even high resolution (electron microscopy) immunocytochemistry is an adequate method for identifying components of this particular cell compartment. The mere presence of the enzymes in the Golgi complex does not in itself exclude their additional presence in secretory vesicles, and neither does it testify to their activity. Note also that the immunocytochemical studies performed to date do not distinguish between the Golgi complex as a whole (as a perinuclear organelle) and the TGN, the most logical conversion compartment.

Another approach has been to separate cell compartments by subcellular fractionation and to assay for conversion enzyme activity, or for the mere presence of the protein by immunoblotting. Once again we are confronted with a technological limitation, since such studies have typically depended upon

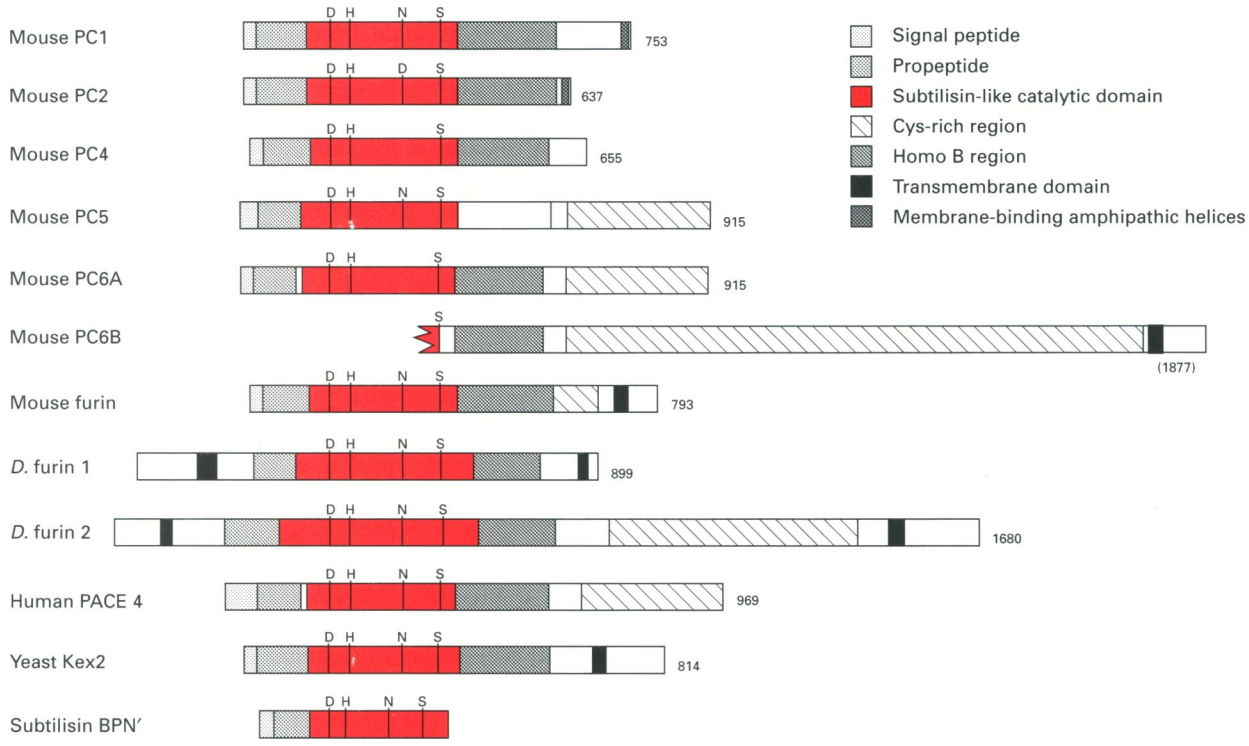

Figure 3 Schematic representation of the subtilisin family members

The structures of a bacterial subtilisin, and of yeast, *Drosophila* (*D.*) and mammalian dibasic processing enzymes are shown. The residues in the subtilisin-like catalytic domain are shown. An alternative, simple uniform terminology for the mammalian enzymes has been proposed by Chan et al. [220] as follows: SPC1, furin (PACE); SPC2, PC2; SPC3, PC1/3; SPC4, PACE 4. It is not clear from this terminology how PC4 should be named. In this formulation, SPC stands for 'subtilisin-related proprotein convertase' or 'secretory pathway convertase' [220].

fractionation of cell homogenates in which it is really not possible to separate with any finesse Golgi vesicles from the constitutive transport vesicles. It will be necessary to use a different approach, and notably one of the techniques for cell-free generation of exocytotic transport vesicles, to determine once and for all whether such vesicles can entertain proprotein conversion.

We are left with the conclusion (unchanged since the albumin studies of the 1970s) that conversion is a late event in the secretory pathway, occurring shortly before exocytosis either in the TGN itself or in secretory vesicles derived from this compartment (and in essence functionally comparable for these purposes).

THE SEARCH FOR THE CONVERSION ENDOPROTEASES

Purification of the enzymes from secretory granule preparations

Shortly after discovering proinsulin and showing that it was the precursor to insulin, Steiner's laboratory was able to mimic the conversion process by partial digestion of proinsulin with trypsin, followed by digestion by carboxypeptidase [165,198]. This seminal observation paved the way for the long search for the trypsin-like conversion endoprotease. For many years this search was hampered by the contamination of secretory granule preparations with lysosomes, leading to the erroneous identification of lysosomal enzymes, such as cathepsins, as the conversion endoprotease.

Despite many attempts to identify the conversion endoprotease, using a variety of different cell or organelle preparations, it was not until 1987 that the long awaited breakthrough was achieved. Studying proinsulin conversion in extracts of granules prepared from insulinoma cells, Hutton and colleagues identified a Ca^{2+}- and acid pH-dependent enzyme activity which met the criteria demanded of a conversion endoprotease [199]. A year later, this same group reported that there were in fact two quite distinct endoprotease activities in their preparation, which they named Type I and Type II [181]. As mentioned above, these two activities differed in their Ca^{2+}- and pH-dependencies, and seemed to display different substrate specificities, in that Type I was able to cleave at the Arg-Arg pair at the proinsulin B-chain/C-peptide junction, whereas the Type II activity cleaved at the other proinsulin conversion site, the Lys-Arg pair linking the C-peptide to the insulin A-chain [181]. The substrate specificity of the conversion endoproteases will be discussed in greater detail below.

The mammalian family of Kex2-like endoproteases

Genetic studies in yeast provided the next breakthrough leading to the identification of a family of eukaryotic conversion endoproteases related to the bacterial subtilisins. The exemplar yeast endoprotease was Kex2, a membrane-bound processing enzyme [200,201]. A computer search for mammalian proteins resembling Kex2 revealed the *fur* (*fes/fps* upstream region) gene and its product furin or PACE [202–204]. More Kex2-related genes were found by PCR amplification based on sequence conservation around the catalytic site, namely PC1/PC3 and PC2 [205–209], PC4 [210,211], PACE 4 [212], PC6A [213], PC6B [214] and PC5 [215] (Figure 3). These endoproteases were found in higher vertebrates, but homologous convertases have also been found in molluscs [216], *Xenopus laevis* [217], *Drosophila melanogaster*

Table 1 Tissue and cellular distribution of the conversion endoproteases

The Table was compiled from the following references: [205,207,209,210,212–215,245,246,253,287].

Source	PC1/3	PC2	PC4	PC5	PC6A	PC6B	Furin	PACE 4
Tissue								
Pituitary	+	+						
Hypothalamus	+	++		+				
Anterior lobe	++	−		+				
Intermediate lobe	+	++		−				
Posterior lobe	−	(−)						
Adrenals	+	(−)		++				
Brain	(−)	++	−		+	−	++	++
Heart	−	−	−		−		++	++
Pancreas	−	−	−		−		+	+
Kidney	−	−	−				++	+
Liver	−	−	−	(+)	−		++	++
Muscle	−	−						+
Lung			−	+	+		++	+
Spleen	−	−		+				
Islet	+	+						
Intestine	−	−	−	++	+	+	++	
Testis			++	(+)	+		++	
Ovaries			−	+				
Placenta								++
Cell line								
AtT20	++	(+)	−	(+)			++	
β-TC3	+	++		(+)				
HepG2	−	−	−		−		++	++
GH3	−	(+)	−	(+)				
GH3 stim	−	+						
BSC-40	−	−	−	(+)	++		+	
LTK−	−	−						
HIns	−	++						
COS	−	−	−		++			
CHO	−	−	−		−		(+)	
3T3	−	−						
PC12	−	−	−		−		++	
RINm5F	(−)	++	−		−			

[218,219] and *Hydra vulgaris* [220]. These convertases have thus been well conserved throughout evolution. In keeping with their proposed role in proprotein conversion, these enzymes correctly cleave precursor proteins at paired basic residues [196,197,221–224]. Kex2, furin and PC6B have hydrophobic transmembrane domains, while PC1, PC2, PC4, PC5, PC6A and PACE 4 do not. PC1 and PC2 have C-terminal amphipathic helices, which in the case of carboxypeptidase E have been shown to associate with intracellular membranes [225] (Figure 3). Furin has been localized in the Golgi membrane [196], but as mentioned above, it remains unclear exactly where it is operational in the cell.

The endoproteases share important sequence similarity in the catalytic domain, and PC2 seems to be the most distant member in evolutionary terms [226] (Figure 3). There is a precise alignment of Asp, His and Ser in the catalytic domains of the different members, although a highly conserved Asn residue has been replaced by Asp in PC2, an interesting mutation since this residue has been shown to play an important role in catalysis in the bacterial subtilisins [227,228]. PC1 and PC2 have an optimal enzyme activity at acidic pH [229–232], while furin has a broad, neutral pH optimum [233]. This family of enzymes has also in common the fact that Ca^{2+} is an essential requirement, although the concentration dependency varies greatly [181]. The two factors, pH and Ca^{2+}, are proposed to modulate enzyme activities and therefore the processing of precursor molecules [181]. Many excellent reviews on the characterization of these endoproteases have appeared [226,234–243], and we shall therefore concentrate on only a few specific aspects of their function, including tissue distribution, post-translational processing of the endoproteases themselves, and cleavage specificity.

Nomenclature of the mammalian conversion endoproteases

In this Review the more commonly used terminology for the mammalian enzymes has been adopted (PC1, PC2 etc.). Note, however, that a new, simplified uniform terminology has been proposed (see legend to Figure 3) based upon the abbreviation 'SPC' and with somewhat different numeration.

TISSUE AND CELLULAR DISTRIBUTION OF THE CONVERTASES

The tissue and cellular distribution of currently identified members of the mammalian Kex2-like family of conversion endoproteases is summarized in Table 1. Furin [244,245], PACE 4 [212] and PC6A [213] are expressed in most tissues and cells so far analysed (albeit at different levels from one cell type to the next). PC6B is expressed mainly in intestine [214], and expression of PC1 and PC2 is restricted to endocrine and neuroendocrine tissues [205,207] including the brain [246–248].

The relative levels of PC1 and PC2 vary widely from one tissue or cell type to the next. PC2 is strongly expressed in mouse

pituitary, hypothalamus and brain, whereas PC1 is very weakly, if at all, expressed in brain. [205,209]. The mRNA distribution in the rat central nervous system has been studied very extensively by *in situ* hybridization [248]. PC1 and PC2 were localized exclusively to neuronal cells, but PC2 was more widely expressed than PC1. Western blot analysis suggests that PC2 is more strongly expressed than PC1 in human insulinoma, while both are strongly expressed in mouse and rat islets [249,250]. A more detailed analysis of expression in rat islet cell subtypes reveals that there is relatively more PC1 in insulin-producing B-cells than in non-B-cells, while the opposite is true for PC2 [251]. Because of this differential expression of the two enzymes in islet B- and non-B-cells it is difficult to interpret the results of studies on the regulation of expression of the two enzymes in islets. It has thus been reported [252] that expression of PC1, but not of PC2, is stimulated by glucose. Since PC2 is expressed principally in non-B-cells, in which glucose has opposing effects to those seen in B-cells, the data could reflect these differences in glucose sensing rather than intrinsic differences in the regulation of expression of the two enzymes.

The expression of PC1 and PC2 in the pituitary gland has been studied in some detail by *in situ* hybridization. PC1 mRNA is strongly expressed in the anterior lobe, while only weak expression of PC2 mRNA can be observed. The opposite is true for the intermediate lobe. No expression of PC1 or PC2 can be observed in the posterior pituitary (Table 1) [205,207,246,253,254]. This is in good agreement with results obtained in AtT20 mouse pituitary corticotroph tumour cells, in which PC1 is much more abundant than PC2 [249]. Indeed, it is not yet clear whether PC2 is expressed at very low levels in these cells, or not at all [249,255]. A more precise co-localization study shows that, in the melanotrophs of the intermediate lobe, PC2 and POMC mRNAs are co-localized. PC1 and POMC have been co-localized in the anterior corticotrophs, while PC2 and POMC mRNAs were rarely observed together. These results are in agreement with the actions of PC1 and PC2 on POMC [224], and with the co-ordinate regulation of POMC, PC1 and PC2 mRNAs that has been demonstrated in the pituitary [253].

PC4 mRNA is expressed exclusively in the testis [210,211], mainly in the early stages of spermatogenesis [210]. One of its possible candidate substrates would be proenkephalin, and indeed PC4 has been co-localized by *in situ* hybridization with proenkephalin [211]. In contrast to PC4, PC5 is expressed at higher levels in female than in male reproductive tissues (Table 1) and in general PC5 exhibits a widespread tissue distribution, although its distribution pattern is quite different from those of PC1, PC2 and furin (Table 1) [215].

POST-TRANSLATIONAL PROCESSING OF THE CONVERTASES

All of the proprotein conversion endoproteases studied to date are synthesized as precursors themselves. The processing of the precursors depends upon cleavage at sites rich in basic residues, and may be either autocatalytic or dependent upon another endoprotease.

Furin

Studies on human furin expression in COS [222,256] or BSC-40 [196,257] cells have shown that the initial precursor of 96–100 kDa is processed to a 90–91 kDa form by autoproteolytic cleavage of the N-terminal pro-region at the consensus furin cleavage site Arg^{104}-Thr-Lys-Arg^{107} [256,257]. The very same cleavage site was identified in CHO cells expressing a mutant furin molecule lacking the transmembrane domain [258]. Mutations of this cleavage site or mutations at the active site Asp^{153} result in the expression only of the larger 96 kDa inactive precursor, showing that removal of the pro-sequence is essential for activation and that the proteolytic maturation is autocatalytic [257]. By expressing active furin together with various mutant furins, these authors confirm that activation occurs by an intramolecular autoproteolytic mechanism. Whether the furin pro-region is important for the proper folding of the enzyme, as is the case for subtilisin E [259–262], has not yet been determined. Further processing at as yet unidentified sites immediately N-terminal to the trans- membrane domain is also known to occur, leading to the generation of soluble (and released) 76–80 kDa forms [222,263]. Soluble and secreted forms of furin have further been shown to be enzymically active [263], and studies on mutant furin molecules have confirmed that the trans-membrane domain is not important for enzymic activity [258].

PC1/PC3 and PC2

The processing of PC1 and PC2 precursors has been studied in some detail in a variety of settings. If there is no consensus as yet on the precise cascade of events or the relevance of processing to the activity of the endoproteases, the essential features are nonetheless apparent.

The PC1 precursor has an apparent molecular mass of approx. 92 kDa in rat islets and is glycosylated (to a 94 kDa form) before processing [252]. An initial cleavage event C-terminal to Arg-Ser-Lys-Arg^{110} in mouse (or Arg^{109} in bovine) PC1 leads to the removal of the N-terminal pro-region residues [185,232,264] and the generation of 80–85 kDa forms [231,264] or an 87 kDa form [232,265]. The discrepancy in size of mouse PC1 reported by the different authors might be due to tissue-specific post-translational modification. These forms are released from GH4C1 and AtT20 cells [231,264,265] but are found within cells only in a membrane-bound form [265]. Further truncation can occur at the C-terminus, leading to the production of several smaller products [266]. In transfected GH4C1 cells such 75, 69 and 60 kDa forms were secreted, but were possibly inactive [231]. In AtT20 cells, by contrast, a 66 kDa product was the major secreted form when secretion was stimulated, and while the 87 kDa form was membrane-associated in the cells, the 66 kDa form was found in soluble and membrane-bound fractions [265]. A similar 66 kDa form is found in rat islets [250,252] and in bovine adrenal medullary secretory granules [185]. Although it is not yet known which, if any, of these events are autocatalytic, or which are catalysed by other proteases such as furin, evidence has been obtained to indicate that furin might not be responsible for cleavage of the pro-segments of PC1 and PC2 [267], which suggests that cleavage might be autocatalytic or that another unidentified proteinase is responsible for this processing [266,267].

The processing of PC2 appears to be limited to the N-terminus, with the initial removal of a propeptide of some 80 residues being reminiscent of the processing of the PC1 precursor. Cleavage of the approx. 75 kDa precursor occurs at Arg-Lys-Arg-Lys^{109} of the bovine [185] and Arg-Lys-Lys-Arg^{108} of the rat [268] PC2 precursor, to yield a 65–66 kDa form. In the rat there appears to be an alternative cleavage site C-terminal to Arg-Gly-Tyr-Arg^{111}. Note that these sequences would be suitable for conversion by furin [233,237]. When PC2 was expressed in *Xenopus* oocytes, it was found that the pro-region was initially cleaved at Lys-Arg-Arg-Arg^{81} to yield an intermediate of 71 kDa, which was further cleaved to a 68 kDa form only after secretion [269]. This unusual processing pattern may be peculiar to this particular, and

unnatural, setting. With this proviso in mind, it is nonetheless interesting to note that in the same study it was found that mutation of Asp^{167}, which lies at the heart of the putative PC2 active site, failed to affect the processing of the PC2 precursor, suggesting that in oocytes such processing is not autocatalytic [269]. It was, finally, also shown that the PC2 precursor was enzymically inert [269].

The kinetics of processing of endogenous PC1 [252] and PC2 [252,270] have been followed in rat islets (the latter in greater detail), and those of PC1 have been studied in AtT20 cells [265]. The processing of both enzymes has also been studied in considerable detail following their expression by vaccinia infection of GH4C1 cells [267]. Processing of PC1 seems to be more rapid than that of PC2 [267]. In GH4C1 cells, it has been proposed that the onset of processing of both the PC1 and PC2 precursors occurs in a pre-Golgi compartment (possibly the RER) [267]. In rat islet cells, a lag period of approx. 60 min precedes the initiation of processing of the PC2 precursor, and the half-time of processing thereafter is of the order of 2 h [270]. These kinetics are quite different from those of proinsulin conversion [252,270]. Indeed, despite the proposed role of PC2 in proinsulin conversion [268], this discrepancy in processing kinetics for enzyme and substrate, taken with the relatively low levels of PC2 in islet B-cells [251], suggest that in the rat islet B-cell it is this latter endoprotease which plays the dominant role [250,251].

CLEAVAGE SPECIFICITIES OF THE ENDOPROTEASES FURIN, PC1 AND PC2

The cleavage specificities of furin, PC1 and PC2 have been studied *in vivo* by co-transfection with substrate precursor proteins into constitutive (COS and BSC-40) or regulated (AtT20 and PC12) cells, or by co-injection into *Xenopus* oocytes. *In vitro*, the cleavage of precursor proteins has been studied using endoproteases purified from secretory granules or from injected oocytes expressing exogenous endoprotease.

Furin

The selectivity of furin for paired basic residues was first shown by demonstrating that co-transfecting furin with pro-vWF [221,222] and β-nerve growth factor (pro-β-NGF) [196] results in mature vWF and active β-NGF. On studying cleavage site mutants of pro-vWF [221,222], a prorenin mutant in which the Pro residue at position -4 had been replaced by Arg [271], or an Arg to Glu mutation at position -4 of the cleavage site of blood clotting factor IX [272], led to the proposal of the consensus sequence motif Arg-Xaa-Lys/Arg-Arg, a sequence which is shared by many precursor proteins released through the constitutive pathway [237]. *In vitro*, the minimal required sequence is Arg-Xaa-Xaa-Arg [233] and, based on cleavage efficiencies of various prorenin mutants, Watanabe et al. have proposed the following rule: Arg at position -1 is essential and in addition at least two out of three basic residues at positions -2, -4, and -6 are required [273]. Cleavage of proteins which are released through the constitutive pathway, such as viral precursors [274–276], proalbumin, complement pro-C3 [197] and the insulin receptor [277], as well as its localization in the Golgi membrane, its activity at neutral pH and its ubiquitous expression (for review see [240]), led to the hypothesis that furin is active in the constitutive pathway.

We have transfected human insulin or rat insulin I and II into hepatoma (FAO) cells containing only the constitutive pathway, and have shown that in these cells human proinsulin is preferentially cleaved at the B-chain/C-peptide junction, while rat insulin II is cleaved only at the C-peptide/A-chain junction [278,279]. Human proinsulin has a basic residue at position -4 only at the B-chain/C-peptide junction, while rat proinsulin II has a -4 basic residue only at the C-peptide/A-chain junction. This cleavage profile corresponds well to the cleavage specificity of furin, which would be a candidate enzyme for processing of proinsulin in constitutively secreting cells. Note, however, that overexpression of furin in COS cells allows for complete conversion of human proinsulin to insulin (F. Vollenweider, J. C. Irminger and P. A. Halban, unpublished work). This stresses the need for caution in interpreting data from studies in which enzymes, or their substrates, are expressed at unphysiological levels.

Aside from the importance of basic residues at, or preceding, the cleavage site, it has been found that the residue immediately C-terminal to the cleavage site also affects conversion. Studies on proalbumin [280] and prorenin [273] have shown that a polar, and preferably an acidic, residue at this site facilitates cleavage.

Although furin clearly prefers paired basic residues at its substrate cleavage site, it has been reported that it is perhaps able to cleave exogenous prosomatostatin in COS-7 and PC12 cells at monobasic sites to somatostatin-28 and antrin [281], albeit at a low efficiency. In the same study these authors show that PC1 is not able to cleave at monobasic sites. Monobasic cleavage, although another important event in proprotein processing, will not be further discussed here (for reviews on this subject see [236,282,283]).

Cleavage specificity of PC1 and PC2 in the processing of POMC

In the anterior lobe of the pituitary, POMC is processed to β-lipotropic hormone (lipotropin; β-LPH), ACTH and *N*-POMC, whereas in the intermediate lobe and brain, further processing of these peptides occurs. ACTH is converted to α-melanocyte-stimulating hormone (α-MSH) and corticotropin-like intermediate lobe peptide (CLIP), and β-LPH to β-endorphin and γ-LPH, whereas *N*-POMC is partially processed to γ-MSH and an N-terminal fragment (Figure 4a). For a review on POMC processing, see [284].

The cleavage specificity of PC1 and PC2 was first demonstrated by their co-transfection with mouse POMC and POMC mutants into the constitutively secreting BSC-40 cells and cell lines derived from endocrine tissue, namely PC12 and AtT20 cells, using the vaccinia expression system [223,224]. PC2 efficiently produced α-MSH and β-endorphin-(1–31), while PC1 generated ACTH and β-LPH. A minor cleavage by PC1 at Lys-Asp-Lys-Arg^{178} was also observed, which generates β-endorphin-(1–31), and cleavage by PC1 at Ala-Gln-Arg-Arg^{76}, which would generate joining peptide (JP), could not be ruled out. Co-expression of mouse PC1 with POMC in *Xenopus* oocytes yields ACTH [285].

In another set of experiments [286], AtT20 cells were transfected with PC2, since these cells express high levels of PC1 but very low levels of PC2 (or none at all). Native AtT20 cells generate β-LPH, ACTH and JP [184] in a strict temporal fashion [286]. Since AtT20 cells have very low levels of PC2, these cleavages are mediated by PC1 and correspond well with the processing pattern observed in the anterior pituitary [284] (Figure 4b), a tissue where PC1 is much more strongly expressed than PC2. When AtT20 cells were transfected with PC2, they were able to perform all the additional cleavages seen in the intermediate pituitary but not in anterior pituitary corticotrophs, to generate β-endorphin-(1–27) and γ_3-MSH. These cleavages were only detected 2 h or more after POMC synthesis. The expression of PC2 also accelerated the production of γ-LPH, β-endorphin-

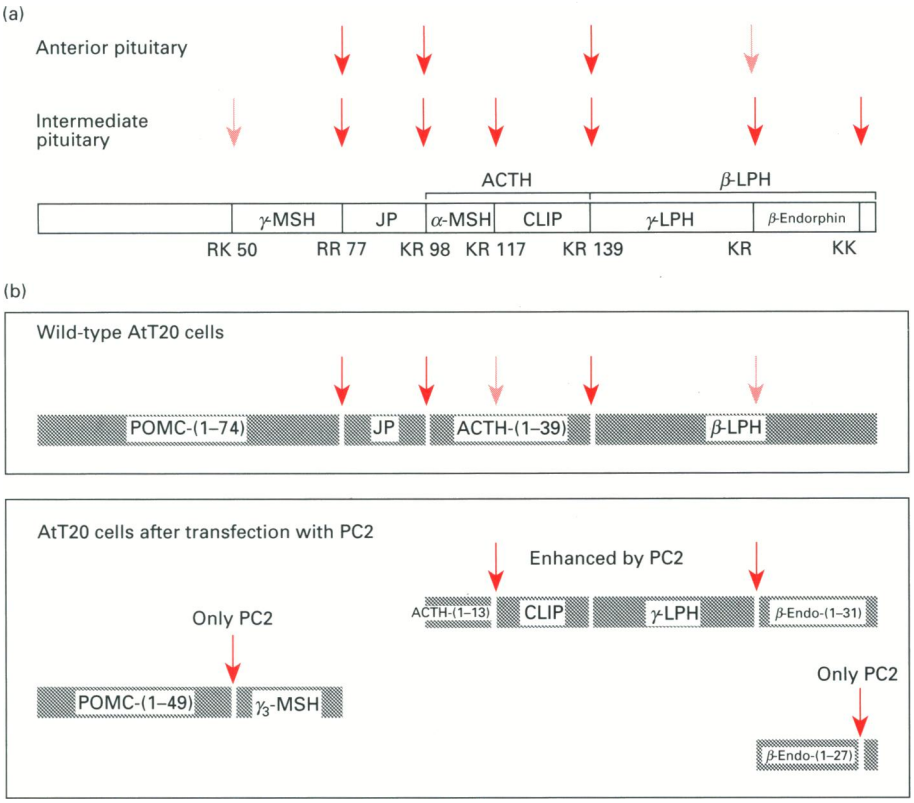

Figure 4 Tissue-specific cleavage of POMC and cleavage specificities of PC1 and PC2

(**a**) Cleavage pattern of POMC in the anterior and intermediate pituitary. (**b**) The upper panel shows the cleavage pattern of POMC in native AtT20 cells. The major products generated are: POMC-(1–74), JP, ACTH-(1–39) and β-LPH. The lower panel shows the additional and enhanced cleavages after AtT20 cells have been transfected with PC2 [286]. Note that the cleavage pattern in native AtT20 cells correlates well with that in the the anterior pituitary, where PC1 is expressed at higher levels than PC2. Transfection of PC2 into AtT20 cells adds the cleavages necessary to generate the products seen in the intermediate pituitary, where PC2 is more abundant than PC1. Red arrows indicate efficient cleaving; pale red arrows indicate partial cleaving.

(1–31), ACTH-(1–13) and CLIP. These cleavages were detected within 1 h after POMC synthesis. The temporal pattern of cleavages in these transformed cells corresponds well with that in the intermediate pituitary, a tissue which has high levels of PC2. Although these results agree with the vaccinia expression data mentioned above, those authors observed neither γ_3-MSH nor β-endorphin-(1–27), products which appear under physiological conditions. These differences could be due to a weakness of the vaccinia expression system, namely a possible disruption of the formation of functional new secretory granules, since normal cellular protein synthesis is shut down by the vaccinia infection. Day et al. reported a much more selective cleavage by PC2 [287]. They have transfected monkey POMC into neuronal cells, which express low levels of PC2 and no PC1. They showed that only β-endorphin is produced, and argue that the cleavage site specificity of PC2 may not have been representative in previous studies, due to the very high level of PC2 obtained with the expression systems used.

Earlier experiments [253] showed that lowering dramatically the expression of PC1, by expressing PC1 antisense RNA in AtT20 cells, resulted in an alteration in the cleavage of endogenous POMC. The cells secreted an unusually large amount of unprocessed POMC and less than normal amounts of the smaller peptide products. Involvement of PC1 and PC2 in tissue-specific differential processing of POMC has been further demonstrated by correlating PC1 and PC2 immunolocalization with cleavage products [288].

Cleavage of mouse POMC by insulin secretory granule activities type I and type II, which have been shown to be most probably identical with PC1 [230,235] and PC2 [268] respectively, was analysed *in vitro*. Only type II Lys-Arg-directed activity cleaved POMC to yield β-endorphin, α-MSH, CLIP and γ-LPH. The tetrabasic Lys-Lys-Arg-Arg[141] in ACTH was cleaved C-terminally of the Lys-Arg sequence. Enigmatically, there was no detectable processing of intact POMC by type I activity alone. Cleavage at the ACTH/β-LPH junction is, however, likely to be due to type I activity associated with type II, since the two activities were not completely separated [289].

These experiments demonstrate that each of the two proteases has a distinct cleavage specificity (although PC2 has a broader range than PC1), thereby accounting for tissue-specific profiles of POMC processing. Although it is likely that PC1 and PC2 are implicated in cleavage of POMC in the pituitary, one has to be aware that there could be additional proteases involved with different cleavage specificities, such as the PC2-like enzyme found in bovine intermediate lobe secretory vesicles [290] or members of the family of aspartic proteases [291,292].

Cleavage specificity of PC1 and PC2 in conversion of proinsulin to insulin

Proinsulin must be cleaved at two discrete sites to release mature insulin and C-peptide. Two possible conversion intermediates are generated by cleavage at just one of these two sites followed,

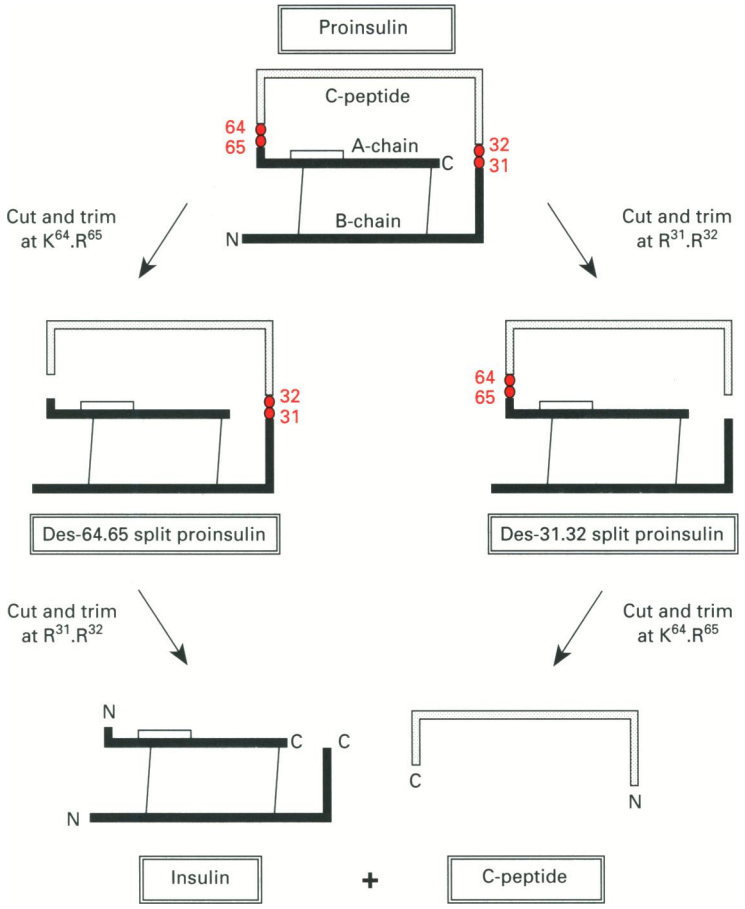

Figure 5 Processing of proinsulin to insulin

The initial cleavage is due to an endoprotease activity, presumably PC1 or PC2, at either the B-chain/C-peptide or the C-peptide/A-chain junction, followed by the trimming of residual C-terminal basic residues by carboxypeptidase H. The two possible processing intermediates are des-31.32 split proinsulin or des-64.65 split proinsulin. A second round of cleavage by PC1 or PC2 and trimming by carboxypeptidase H generates mature insulin and C-peptide.

by trimming of C-terminal basic residues by carboxypeptidase H [293], namely des-31.32 and des-64.65 split proinsulin (Figure 5). As discussed above, in rat insulinoma granules a type I activity cleaves at the Arg-Arg sequence at the B-chain/C-peptide junction, and a type 2 activity cleaves at the Lys-Arg sequence at the C-peptide/A chain junction [181,294]. It has been shown that type II activity corresponds to PC2 [268], and there is good evidence that type I activity is identical with PC1 [230,235,250]. Several independent studies have shown the importance of basic residues in proinsulin conversion. The incorporation of Lys and Arg analogues into newly synthesized proinsulin thus leads to the inhibition of conversion [295]. More specifically, mutation of the dibasic residues [294] and the use of active-site-directed peptides [296] has shown that type 1 (PC1) and type 2 (PC2) enzymes require the presence of pairs of basic residues at their respective proinsulin cleavage sites. Finally, it has been shown that in some patients with familial hyperproinsulinaemia in which one of the basic amino acids at one of the cleavage sites has been altered, a partially cleaved proinsulin conversion intermediate is found in the circulation [297].

The cleavage specificity of proinsulin by PC1 and PC2 has been analysed in more detail by co-transfection of rat insulin I into COS cells with human PC1 and mouse PC2 [249]. PC1 generates mature insulin but cleaves preferentially at the B-chain/C-peptide junction, while PC2 selectively cleaves at the C-peptide/A-chain junction. The data for PC2 activity in COS cells are in agreement with the specificity of type II activity studied *in vitro* [181]. Intriguingly, whereas PC1 appears able to cleave proinsulin at both junctions in COS cells, it can only cleave at the B-chain/C-peptide junction *in vitro* [230], and a similar selective substrate specificity has been shown for the type I enzyme *in vitro* [181]. It is, however, difficult to compare the *in vivo* and *in vitro* studies. Rat proinsulin I (with a -4 basic residue preceding both cleavage sites) was the substrate in COS cells, whereas human proinsulin (-4 basic residue only preceding the B-chain/C-peptide junction) was used for the *in vitro* studies. By comparing the kinetics of conversion of both rat proinsulins and human proinsulin in isolated islets, we have shown that a -4 basic residue is important for cleavage at both junctions [298,299]. One must also take into consideration that the COS cells have no regulated pathway and that PC1 and PC2 are therefore operating under unusual circumstances in these cells, in addition to being expressed at unphysiologically high levels in this transfection setting.

The basic residues at positions -1, -2 and -4 are most probably not the only factor involved in determining the substrate specificity of these endoproteases. Deletion of the highly conserved first four residues of the C-peptide in rat insulin inhibits conversion of proinsulin [175]. Whether this is due to the importance of all these residues in the recognition of the cleavage

site by the endoprotease, or to the fact that deletion of the residues brings a proline rather than a glutamate C-terminal to the dibasic residues, is under current investigation in our laboratory.

The importance of secondary and tertiary structure in proprotein processing

Evidence for the importance of substrate structural domains in determining the activity of the endoproteases has been reported by several groups. The idea that such structures might play a role in the definition of substrate binding sites and therefore in processing enzyme recognition was proposed by Geisow and Smyth [300,301]. The comparison of the structure of 20 different prohormone sequences with 53 dibasic potential cleavage sites [302] has shown that dibasic sites located in or next to β-turns were cleaved, whereas sites situated in ordered structures like β-sheets or α-helices were not. Using the pro-oxytocin/neurophysin processing domain and its putative convertase as a model, it was shown that processing at dibasic sites is associated with a sequence organized in a β-turn structure. The β-turn is an interchangeable domain, since it can be replaced by another sequence able to form such a motif, and it is suggested that this secondary structure participates in favouring the interaction of the substrate with the processing enzyme [303,304].

Structural domains have also been implicated in proinsulin processing [72,131]. Mutating Arg^{31}-Arg^{32} to Arg^{31}-Gly^{32} at the B-chain/C-peptide junction [294] leads to a substrate which is not cleaved by type I activity but is also a poor substrate for type II activity which, as described above, has been shown to cleave specifically at the C-peptide/A-chain junction. Preferential cleavage of des-31.32 split proinsulin over intact proinsulin by type II endopeptidase has also been reported [305], suggesting a structural constraint inhibiting cleavage at the C-peptide/A-chain junction which might be relieved by initial cleavage at the B-chain/C-peptide junction [305]. A potentially important structural domain in proinsulin, the CA knuckle, has been defined based upon n.m.r. studies of the molecule [306]. This domain may also be implicated in cleavage at the C-peptide/A-chain junction, but this has yet to be tested directly.

SUMMARY AND FUTURE PROSPECTS

Many of the key features underlying the intracellular trafficking and processing of secretory proteins are now understood in quite some detail. All secretory proteins are transported to the TGN without any apparent prior sorting. Once in the TGN, an active sorting event is responsible for targeting proteins to the regulated pathway. Exit from the TGN to the constitutive pathway is by default. Proproteins are processed (converted) in both secretory pathways by endoproteolysis at sites typically presenting two, and often more, basic residues. The enzymes responsible for conversion are members of a mammalian family of endoproteases closely related to the yeast Kex2 endoprotease. The tissue distribution of these enzymes suggests that whereas some (i.e. furin and PACE 4) are ubiquitous and most probably responsible for conversion in the constitutive pathway, others present a more restrictive pattern of expression. PC1 and PC2 are expressed only in cells which have the regulated pathway. Based upon this and other biochemical criteria, it is now understood that these are the prohormone conversion endoproteases.

A number of questions remain unanswered, and will surely be the focus of research over the next few years.

The sorting mechanism

Despite considerable advances in our understanding of how proteins are recognized, concentrated and targeted to nascent granules in the TGN, the precise mechanism remains elusive. Future studies will hopefully show once and for all whether a receptor or receptor-like molecule is indeed involved, and if so one can expect such a molecule (or molecules) to represent a new class of receptor with quite broad specificity. If self-association of regulated proteins with the TGN, rather than a receptor-mediated event, is involved, the missing link responsible for targeting and association of clathrin must be identified. Finally, although it is now quite apparent that concentration of some proteins can arise by condensation, the physico-chemical nature of the condensation event is far from understood. Indeed, once in a condensed state, how can the proteins be converted? (As Steiner has indicated [307], a condensed protein is not likely to be an attractive substrate for a conversion endoprotease, yet condensation is typically poorly reversible.)

The conversion endoproteases

The tissue-specific regulation of expression of the conversion endoproteases PC1 and PC2 clearly dictates cellular patterns of prohormone conversion. It will be intriguing to learn more about the *cis*-elements and cognate *trans*-factors implicated in such regulation of expression, and see to what extent there is any overlap with the regulation of expression of the genes encoding the substrates for the endoproteases, i.e. the prohormones themselves. One clue to such overlap lies in the observation that the expression of both PC1 and proinsulin are regulated by glucose in islets.

Since both PC1 and PC2 must find their way to the granules of the regulated pathway, the issue of sorting in the TGN is as pertinent to these enzymes as it is to prohormones. Indeed, studying the sorting of these enzymes may provide clues to understanding the sorting process as a whole.

In the future, in all probability, new members of this growing family of convertases will be discovered and characterized. It will be necessary to examine more precisely the substrate/enzyme interactions which determine recognition and cleavage specificity of the newly discovered endoproteases and those already known. It will also be important to study in greater detail their structural characteristics and the mechanisms involved in their own post-translational processing. With such knowledge, this rapidly evolving field will certainly have an important impact on biotechnology and medicine.

Differentiation of regulated cells

The sorting and conversion machinery of regulated secretory cells are obviously unique features of their differentiated function. What else is needed to endow a cell with this remarkable pathway? Granules carry both inside them, in soluble form, and on their membrane, a myriad of proteins. We must try to understand how each of them reaches the granule, and their role in granulogenesis and exocytosis.

Note added in proof (received 7 February 1994)

The reader is referred to refs. [308] and [309], which appeared after completion of this review, for additional information on the processing of pro-PC1 and pro-PC2.

Our work is supported by grant no. DK-35292 from the N. I. H. and by a grant from Hoechst AG. This laboratory is a member of the Geneva Diabetes Group. We thank

M. Neerman-Arbez and Dr. F. Vollenweider for their useful comments and help in preparing this review.

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